APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1995, p. 1274–1278 Vol. 61, No. 4
0099-2240/95/$04.00+0

Amplification of DNA Polymerase Gene Fragments from Viruses Infecting Microalgae†

FENG CHEN AND CURTIS A. SUTTLE*

Department of Marine Science, University of Texas at Austin, Port Aransas, Texas 78373-1267

Received 17 October 1994/Accepted 20 January 1995

Nested PCR with three highly degenerate primers was used for amplification and identification of DNA polymerase (*pol*) genes from viruses which infect three genera of microalgae. Group-specific primers (AVS1 and AVS2) were designed on the basis of inferred amino acid sequences unique to the DNA *pol* genes of viruses (PBCV-1 and NY-2A) that infect an endosymbiotic *Chlorella*-like alga (*Chlorophyceae*) and a virus (MpV-SP1) which infects the photosynthetic flagellate *Micromonas pusilla* (*Prasinophyceae*). In addition, a nested primer (POL) was designed on the basis of the highly conserved amino acid sequence YGDTDS found in most B-family (α-like) DNA *pol* genes. These primers were used to amplify DNA from the three viruses, PBCV-1, NY-2A, and MpV-SP1, for which the primers were designed, as well as eight clonal isolates of genetically distinct viruses which infect *M. pusilla* and others which infect *Chrysochromulina* spp. (*Prymnesiophyceae*), suggesting that these are a group of related viruses. In contrast, no product resulted from using DNA from viruses which infect the marine brown algae *Ectocarpus siliculosis* and *Feldmannia* sp. (*Phaeophyceae*), suggesting that these viruses may not be closely related to those that infect microalgae. These primers were also used to amplify DNA from natural virus communities. Our results indicate that nested PCR, even under low-stringency conditions, can be used as a rapid method to verify the presence in seawater of a group of related viruses which infect microalgae. Sequence analysis of these fragments should provide information on the genetic diversity and potentially the phyletic relationships among these viruses. This is the first example of a PCR-based technique designed to detect viruses which infect eukaryotic algae.

The abundance of virus particles in the sea ranges from ca. 10^6 to 10^8 ml^{-1}, and data suggest that they are of considerable ecological importance to natural communities of bacteria and cyanobacteria (reviewed in references 5, 7, 13, and 35). Viruses which infect eukaryotic algae have received less attention. However, recent studies suggest that they also may be important agents of mortality for phytoplankton, thereby affecting community structure and flow of nutrients and energy in aquatic systems (7, 35, 37). Virus-like particles are known to occur in at least 44 taxa of eukaryotic algae; however, except for a few cases, particles have not been isolated or purified (42).

Most of our knowledge about viruses which infect eukaryotic algae comes from the few virus-host systems isolated and purified in the laboratory. The most substantial work has been done on those viruses which infect freshwater *Chlorella*-like algae that are endosymbionts of *Paramecium bursaria* (26, 40, 42) and on viruses which infect the marine photosynthetic flagellate *Micromonas pusilla* (11, 24, 25, 44). Studies have demonstrated that the viruses which infect a *Chlorella* sp. and *M. pusilla* are geographically widespread and morphologically similar but are genetically different (11, 12, 46). Considerable work has also been done on viruses which infect the filamentous brown algae *Ectocarpus siliculosis* (22, 28–30) and *Feldmannia* sp. (17), and infected plants have been found to occur over a wide geographic range (30).

Despite their potential importance, little is known about viruses which infect microalgae other than those which infect *Chlorella*-like endosymbionts, and even less is known about the evolutionary relationships among them. Moreover, enumeration of viruses that infect microalgae in natural samples is difficult because morphological criteria which allow them to be distinguished by electron microscopy do not exist. Even if objective morphological criteria could be defined, their abundance would probably be too low to be counted by this method. Currently, viruses which infect phytoplankton are typically counted by most-probable-number assay (11, 36); hence, only viruses for which hosts have been isolated can be enumerated. Furthermore, it can take days or weeks to obtain estimates of abundance by these approaches. Consequently, there is a need to develop methods to detect, identify, and quantify viruses which infect phytoplankton.

One approach is to develop algal virus-specific PCR primers. PCR has been used to detect a variety of viruses in aquatic environments, including hematopoietic necrosis virus which infects salmon and trout (3), enteroviruses (21, 39), hepatitis A virus (39), and human immunodeficiency virus (2). A primary consideration in developing a PCR-based method for detecting microalgal viruses was to identify a suitable gene for which primers could be designed.

Over 50 DNA *pol* genes from a variety of organisms and viruses have been isolated and sequenced. Eukaryotes, prokaryotes, and some viruses possess B-family (α-like) DNA polymerases which contain exonuclease and polymerase domains. These motifs are highly conserved at the amino acid level but not necessarily at the nucleotide level; hence, DNA *pol* genes are ideal targets for which PCR primers of wide-ranging specificity can be designed (47). Within the polymerase domain, the catalytic site YGDTDS is the most highly conserved and is a good target for gene probing (6, 16, 18, 43) (Fig. 1A). The DNA *pol* genes from two viruses (PBCV-1 and NY-2A) which infect a *Chlorella*-like alga symbiotic with *P. bursaria*

* Corresponding author. Mailing address: Department of Marine Science, University of Texas at Austin, P.O. Box 1267, Port Aransas, TX 78373-1267. Phone: (512) 749-67331. Fax: (512) 749-6777.

† Contribution number 930 of the Marine Science Institute.

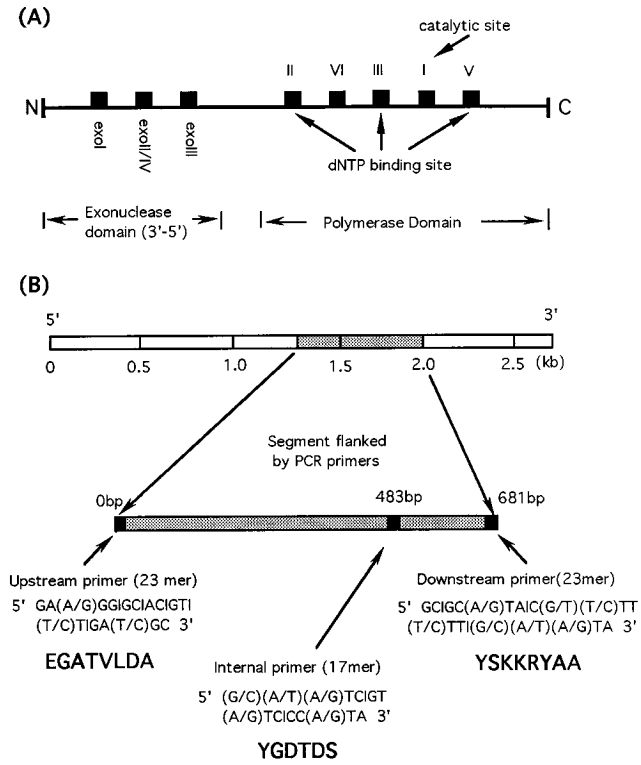

FIG. 1. (A) Generic map of B-family (α-like) DNA polymerases, showing conserved motifs and their possible functions (modified from reference 38). The most highly conserved amino acid sequence, YGDTDS, is located in motif I (catalytic site). dNTP, deoxynucleoside triphosphate. (B) Relative positions of the three primers on the DNA *pol* gene. The upstream and downstream primers (AVS1 and AVS2) are specific for algal viruses, and the nested primer (POL) is universal for B-family DNA polymerases.

have been sequenced. These viruses have recently been shown to belong to a newly recognized family of viruses, *Phycodnaviridae* (41). In addition, the DNA *pol* gene from a virus (MpV-SP1) which infects the photosynthetic picoflagellate *M. pusilla* has been sequenced and compared with other known DNA *pol* genes in the GenBank database (47). Several inferred amino acid sequences were completely conserved among MpV-SP1, PBCV-1, and NY-2A but were not found in any other DNA *pol* sequences in the GenBank database (47). In addition, high similarities among the inferred amino acid sequences of these viruses suggest that viruses which infect these algae may be closely related. From this sequence information, we designed two degenerate PCR primers which can be used to amplify DNA from this group of related viruses, and a third primer which can be used with nested PCR to confirm that the target sequence has been amplified. The motivation was to design primers suitable for amplifying DNA from viruses that infect eukaryotic microalgae.

MATERIALS AND METHODS

Viruses and DNA extraction. Nine genetically distinct clonal isolates of MpV, viruses which cause lysis of *M. pusilla*, were isolated (11) from coastal waters of New York (MpV-PB5, -PB6, -PB7, and -PB8), Texas (MpV-PL1), California (MpV-SP1 and -SP2), and British Columbia (MpV-SG1), as well as from oligotrophic water of the central Gulf of Mexico (MpV-GM1) (Table 1). The protocols for isolation and amplification of MpV and purification of DNA can be found elsewhere (11). DNA from viruses PBCV-1 and NY-2A, which infect a *Chlorella*-like endosymbiont (42), was graciously provided by Y. P. Zhang and J. L. Van Etten. DNA from viruses that infect the brown algae *E. siliculosis* (29) and *Feldmannia* sp. (17) was graciously provided by D. G. Müller and S. T. J. Lanka, and R. H. Meints, respectively. DNA was also extracted from two clonal isolates of viruses (CbV-PW3) and CbV-LM1) which infect *Chrysochromulina* spp. (36) and from two natural marine virus communities (VC102 and VC112) which were concentrated by ultrafiltration from coastal waters of Texas (38).

Primer design. To maximize the probability of amplifying DNA *pol* gene fragments from viruses related to PBCV-1, NY-2A, and MpV-SP1, a pool of primers which corresponded to each amino acid sequence was used to compensate for degeneracy in the genetic code. Deoxyinosine was also used at positions of complete degeneracy to reduce the size of the oligonucleotide pool (4, 20). Three oligomers (AVS1, AVS2, and POL) were used (Fig. 1B). The upstream primer (AVS1) and downstream primer (AVS2) correspond to the amino acid sequences EGATVLDA and YSKKRYAA, respectively. They were completely conserved among these three algal viruses and are highly specific on the basis of a search of the GenBank database. Calculated melting temperatures for AVS1 (5′-GA[A/G]GGIGCIACIGTI[T/C]TIGA[T/C]GC-3′) and AVS2 (5′-GCIGC [A/G]TAIC[G/T][T/C]TT[T/C]TTI[G/C][A/T][A/G]TA-3′) were 66 to 72 and 56 to 66°C, respectively, assuming 4°C for G or C and 2°C for A, T, or I. The lengths of fragments amplified by this set of primers should be 683, 808, and 793 bp for MpV-SP1, PBCV-1, and NY-2A, respectively. Second-step PCR amplification with AVS1 and nested primer POL (5′-[G/C][A/T][A/G]TCIGT[A/G]TCICC [A/G]TA-3′; melting temperature, 46 to 52°C), corresponding to the amino acid sequence YGDTDS, was used to confirm that these products were amplified from DNA *pol* genes. AVS1-POL amplicons from second-step PCR were predicted to range from 506 to 533 bp on the basis of DNA sequences for MpV-SP1, PBCV-1, and NY-2A. The upstream, downstream, and nested primers were 23-, 23-, and 17-mers and had degeneracies of 8,192-, 4,096-, and 512-fold, respectively.

Nested PCR. Virus DNA (100 ng) was added to 25 μl of a PCR reaction mixture which contained *Taq* DNA polymerase assay buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 0.1% Triton X-100, 1.5 mM $MgCl_2$), 0.16 mM each deoxyri-

TABLE 1. Descriptions of viruses used in this study

Host species	Virus	Morphological characteristics[a]	Sample source and/or location[b]	Reference
M. pusilla	MpV-SP1	dsDNA, polyhedral, 104- to 118-nm diam	Scripps pier, Calif. (32°45′N, 117°15′W)	9
	MpV-SP2		Scripps pier, Calif. (32°45′N, 117° 15′W)	
	MpV-PB5		Peconic Bay, N.Y. (40°00′N, 72°30′W)	
	MpV-PB6		Peconic Bay, N.Y. (40°00′N, 72°30′W)	
	MpV-PB7		Peconic Bay, N.Y. (40°00′N, 72°30′W)	
	MpV-PB8		Peconic Bay, N.Y. (40°00′N, 72°30′W)	
	MpV-PL1		MSI pier, Tex. (27°50′N, 97°04′W)	
	MpV-SG1		Strait of Georgia (49°18′N, 124°11′W)	
	MpV-GM1		Gulf of Mexico (26°20′N, 89°59′W)	
Chlorella strain NC64A	PBCV-1	dsDNA, polydedral, 150- to 190-nm diam	Culture media	37
	NY-2A		Unknown river in New York	
Chrysochromulina brevifilum	CbV-PW3	dsDNA, polyhedral, 145- to 170-nm diam	MSI pier, Tex. (27°50′N, 97°04′W)	This study
	CbV-LM1		Laguna Madre (27°30′N, 97°18′W)	
Natural communities	VC102	Unknown	MSI pier, Tex. (27°50′N, 97°04′W)	This study
	VC112		MSI pier, Tex. (27°50′N, 97°04′W)	

[a] dsDNA, double-stranded DNA.
[b] MSI, Marine Science Institute.

bonucleoside triphosphate, 30 pmol of each primer, and 0.625 U of *Taq* DNA polymerase (Promega). Mineral oil (20 μl) was added to each reaction tube. Negative controls contained all reagents except template. PCR was conducted with a Minicycler (MJ Research) according to the following cycle parameters: denaturation at 95°C (30 s), annealing at 50°C (45 s), and extension at 72°C (1 min). Following 30 rounds of amplification, PCR products were electrophoresed on 1% SeaPlaque GTG agarose (FMC BioProducts, Rockland, Maine) in 0.5× TBE buffer (0.045 M Tris-borate, 1 mM EDTA [pH 8.0]) and examined by ethidium bromide staining. For second-step amplification, agarose plugs which contained templates were excised from gels with Pasteur pipettes and then combined with PCR mixtures (27, 49). All of the reaction mixtures and conditions for second-step PCR were the same as those for first-step amplification, except that the downstream primer was replaced by the nested primer. AVS1-POL amplicons were run on 1% SeaKem LE agarose (FMC BioProducts) in 0.5× TBE buffer and visualized as described above.

RESULTS

PCR amplification with the primer pair AVS1 and AVS2 generated ca. 680-bp bands for all nine clones of MpV and ca. 800-bp bands for PBCV-1 and NY-2A (Fig. 2A). Nested PCR with the primer pair AVS1 and POL showed bands of ca. 500 bp for all nine clones of MpV and bands of ca. 530 bp for PBCV-1 and NY-2A (Fig. 2B). No products were observed for negative controls during both rounds of PCR (Fig. 2A and B, lanes 13). Fragments amplified by the AVS1 and AVS2 primers from the *Chrysochromulina* viruses (CbV-PW3 and CbV-LM1) and DNA from natural virus communities (VC102 and VC112) also corresponded to ca. 680 bp. Products were not obtained with DNA from the viruses which infect *E. siliculosis* and *Feldmannia* sp. PCR with primers AVS1 and AVS2 was also run against DNA extracted from *M. pusilla* and two bacteriophages isolated from coastal waters of Texas, and no products were detected (data not shown). All AVS1-AVS2 amplicons were reamplified by primers AVS1 and POL, resulting in products of approximately 500 bp (Fig. 2C). The AVS1-AVS2 and AVS1-POL amplicons of CbV-PW3 and CbV-LM1 appeared to be slightly larger than those from MpV clones; this will be confirmed by sequencing PCR products at a later date.

DISCUSSION

The approach presented here can be used to amplify segments of DNA *pol* genes from a group of viruses which infect microalgae. Although the degeneracies of primers were as high as 8,192-fold, we still achieved the desired specificity and sensitivity. No nonspecific amplification was found even if host and phage DNA was added to reactions. Mack and Sninsky (23) used PCR with degenerate primers (8- and 256-fold) to amplify uncharacterized viruses related to known virus groups in the hepadnavirus model system, while Knoth et al. (20) used primers with degeneracies as high as 1,024-fold to amplify rare cDNA. Others have used primers with degeneracies of 10^5 to 10^6 or even greater to amplify desired gene segments from distantly related species (14, 15).

Nested PCR has been used as a rapid method for confirming PCR products (1, 19, 27, 34, 48). Conventionally, Southern hybridization is used to confirm that the correct product has been amplified; this may take several days to complete. In contrast, results can be obtained within a few hours by using nested PCR. In our case, second-step PCR was run with the original upstream primer and the probe primer (8, 9). Kai et al. (19) suggested that PCR with three primers was useful for confirming amplified products; however, it was not shown that nested PCR works when the degeneracies of primers are high and only three primers are involved. Our results demonstrate that nested PCR still provides enough sensitivity and specificity, even if three highly degenerate primers are used and second-step amplification is performed in low-melting-point agarose, because first-step PCR provides millionfold increases in the designated regions and also exponentially reduces nucleic acid complexity (23). The approach developed here allowed us to amplify and then quickly identify segments of DNA *pol* genes from a variety of viruses which infect microalgae. The same approach should be suitable for use in other systems in which only one internal site is available. It has advantages over the traditional hybridization method in terms of safety, cost, and simplicity.

The group-specific primers AVS1 and AVS2 were originally derived from inferred amino acid regions on the basis of sequence analysis of the entire DNA *pol* genes of two viruses which infect a *Chlorella*-like alga and of a virus which infects *M. pusilla*. Using this primer pair, we successfully amplified DNA

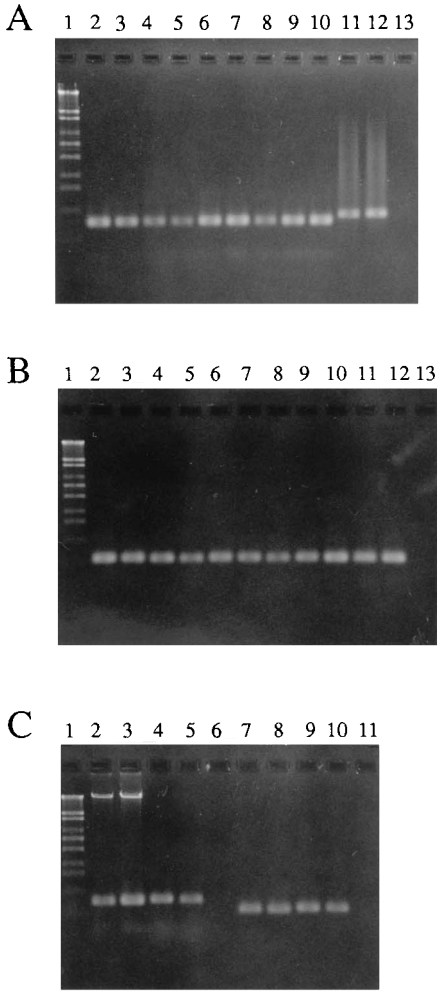

FIG. 2. Analysis of PCR products by agarose gel electrophoresis. Lanes 1, molecular weight marker (phage lambda DNA digested with *Sty*I, with bands corresponding to 19,329, 7,743, 6,223, 4,254, 3,472, 2,690, 1,882, 1,489, 925, and 421 bp, respectively). (A) Fragments from the nine virus isolates which infect *M. pusilla* and the two virus isolates which infect a *Chlorella*-like alga were amplified by the primer set AVS1 and AVS2. Lanes 2 to 10, MpV-SP1, -SP2, -PB5, -PB6, -PB7, -PB8, -GM1, -SG1, and -PL1, respectively; lanes 11 and 12, PBCV-1 and NY-2A, respectively; lane 13, negative control. (B) The amplicons shown in panel A were excised and reamplified by the nested primer pair AVS1 and POL. Lanes are the same as indicated for panel A. (C) PCR products from CbV-PW3 and CbV-LM1 and DNA extracted from natural virus communities VC102 and VC112. Lanes 2 to 5, PCR products of VC102, VC112, CbV-PW3, and CbV-LM1, respectively, amplified by AVS1 and AVS2; lanes 7 to 10, amplicons of AVS1 and AVS2 from lanes 2 to 5 (in the same order) excised and reamplified by the nested primer set AVS1 and POL; lanes 6 and 11, negative controls.

from clonal isolates of viruses that infect three unrelated microalgae (*M. pusilla*, an endosymbiotic *Chlorella*-like alga, and *Chrysochromulina* spp.), implying that these three groups of viruses are probably related. In contrast, we were unable to amplify DNA from the viruses which infect *E. siliculosis* and *Feldmannia* sp., suggesting that these viruses are not closely related to those which infect microalgae. These primers were used to amplify DNA extracted from natural virus communities concentrated from seawater; therefore, the primers should be useful for obtaining sequence information directly from natural virus populations. The AVS1-AVS2 amplicons from PBCV-1 and NY-2A were larger than those from the viruses that infect *M. pusilla* because the AVS1 and AVS2 primers flank small introns of 101 and 86 nucleotides on the PBCV-1 and NY-2A DNA *pol* genes, respectively (16).

The method presented here provides a powerful tool for rapid amplification and identification of DNA from viruses which infect microalgae. Moreover, by sequence analysis of PCR fragments, it should be possible to infer a gene phylogeny among these viruses. Preliminary analysis of partial sequences of these gene fragments indicates that it is possible to resolve evolutionary relationships both among viruses that infect different algal classes and within viruses that infect a single class (10). Taxon-specific PCR primers should work equally well for detecting, amplifying, and identifying DNA *pol* genes from other organisms and viruses. B-family DNA polymerases are found in eukaryotes, prokaryotes, and some double-stranded DNA viruses.

PCR has been used to amplify *nif* (45), *rbcL* (33), and *rpoC1* (31, 32) genes from aquatic environments. Nested PCR with nondegenerate primers has also detected adenoviruses and enteroviruses in polluted water (34). Our data extend these results to show that it is possible to amplify DNA *pol* gene segments from natural virus communities concentrated from seawater by ultrafiltration even if highly degenerate primers are used. This should permit inferences to be made on the genetic diversity of phycodnaviruses in nature.

Although many virus-like particles have been observed in numerous classes of algae over the past 20 years, few have been isolated and purified. Hence, most viruses associated with algae have remained poorly characterized. Furthermore, the lack of a biological assay for many algal viruses has made it difficult to study them in natural aquatic environments. Study of the dynamic interactions between algae and their viruses in natural ecosystems requires a simple, sensitive, and accurate technique. The approach we have described will allow additional sequence information to be obtained for viruses which infect eukaryotic algae. This will permit us to design primers with less or no degeneracy that are specific for particular groups of algal viruses. Ultimately, this may allow the development of more sensitive PCR methods which can be used to detect and quantify specific algal viruses in natural environments.

ACKNOWLEDGMENTS

We thank H. R. Deyoe for help with PCR, M. T. Cottrell for providing MpV DNA, and A. M. Chan for providing *Chrysochromulina* viruses. DNA from viruses that infect *Chlorella* sp., *E. siliculosis*, and *Feldmannia* sp. were supplied by J. C. Van Etten and Y. P. Zhang of the University of Nebraska at Lincoln, D. G. Müller and S. T. J. Lanka of the University of Constance, and R. H. Meints of Oregon State University, respectively. The comments of referees improved the manuscript.

This work was supported by grant N00014-92-J-1676 from the U.S. Office of Naval Research and an E. J. Lund Scholarship awarded to F. Chen from the University of Texas at Austin.

REFERENCES

1. **Albert, J., and E. M. Fenyö.** 1990. Simple, sensitive, and specific detection of human immunodeficiency virus type 1 in clinical specimens by polymerase chain reaction with nested primers. J. Clin. Microbiol. **28:**1560–1564.
2. **Ansari, S. A., S. R. Farrah, and G. R. Chaudhry.** 1992. Presence of human immunodeficiency virus nucleic acids in wastewater and their detection by polymerase chain reaction. Appl. Environ. Microbiol. **58:**3984–3990.
3. **Arakawa, C. K., R. E. Deering, K. H. Higman, K. H. Oshima, P. J. O'Hara, and J. R. Winton.** 1990. Polymerase chain reaction (PCR) amplification of a nucleoprotein gene sequence of infectious hematopoietic necrosis virus. Dis. Aquat. Org. **8:**165–170.
4. **Bartl, S., and I. L. Weissman.** 1994. PCR primers containing inosine triplet to complement a variable codon within a conserved protein-coding region. BioTechniques **16:**246–250.
5. **Børsheim, K. Y.** 1993. Native marine bacteriophages. FEMS Microbiol. Ecol. **102:**141–159.
6. **Braithwaithe, D., and J. Ito.** 1993. Compilation, alignment, and phylogenetic relationships of DNA polymerases. Nucleic Acids Res. **21:**787–802.
7. **Bratbak, G., F. Thingstad, and M. Heldal.** 1994. Viruses and the microbial loop. Microb. Ecol. **28:**209–221.
8. **Chen, F., M. T. Cottrell, and C. A. Suttle.** 1994. Development of a PCR-based technique for detecting and quantifying algal viruses in aquatic environments, p. a–16. *In* ASLO & PSA 1994 Joint Meeting. American Society for Limnology and Oceanography, Lawrence, Kans.
9. **Chen, F., and C. A. Suttle.** Nested PCR with three highly degenerate primers for amplification and identification of DNA from related organisms. BioTechniques, in press.
10. **Chen, F., and C. A. Suttle.** Unpublished data.
11. **Cottrell, M. T., and C. A. Suttle.** 1991. Wide-spread occurrence and clonal variation in viruses which cause lysis of a cosmopolitan, eukaryotic marine phytoplankter, *Micromonas pusilla*. Mar. Ecol. Prog. Ser. **78:**1–9.
12. **Cottrell, M. T., and C. A. Suttle.** 1994. Comparison of viruses that lyse the marine photosynthetic flagellate *Micromonas pusilla* using quantitative DNA-DNA hybridization, p. a–19. *In* ASLO & PSA 1994 Joint Meeting.American Society for Limnology and Oceanography, Lawrence, Kans.
13. **Fuhrman, J. A., and C. A. Suttle.** 1993. Viruses in marine planktonic systems. Oceanography **6:**51–63.
14. **Gonzalez, G. A., K. K. Yamamoto, W. H. Fischer, D. Karr, P. Menzel, W. Biggs III, W. W. Vale, and M. R. Montiminy.** 1989. A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. Nature (London) **337:**749–752.
15. **Gould, S. J., S. Subramani, and I. E. Scheffler.** 1989. Use of the DNA polymerase chain reaction for homology probing: isolation of partial cDNA or genomic clones encoding the iron-sulfur protein of succinate dehydrogenase from several species. Proc. Natl. Acad. Sci. USA **86:**1934–1938.
16. **Grabherr, R., P. Strasser, and J. L. Van Etten.** 1992. The DNA polymerase gene from *Chlorella* viruses PBCV-1 and NY-2A contains an intron with nuclear splicing sequences. Virology **188:**721–731.
17. **Henry, E. C., and R. H. Meints.** 1992. A persistent virus infection in *Feldmannia* (Phaeophyceae). J. Phycol. **28:**517–526.
18. **Ito, J., and D. K. Braithwaite.** 1991. Compilation and alignment of DNA polymerase sequences. Nucleic Acids Res. **19:**4045–4057.
19. **Kai, M., S. Kamiya, S. Sawamura, T. Yamamoto, and A. Ozawa.** 1991. Simplified method for confirmation of PCR products. Nucleic Acids Res. **19:**4562.
20. **Knoth, K., S. Roberds, C. Poteet, and M. Tamkun.** 1988. Highly degenerate, inosine-containing primers specifically amplify rare cDNA using the polymerase chain reaction. Nucleic Acids Res. **22:**10932.
21. **Kopecka, H., S. Dubrou, J. Prevot, J. Marechal, and J. M. López-Pila.** 1993. Detection of naturally occurring enteroviruses in waters by reverse transcription, polymerase chain reaction, and hybridization. Appl. Environ. Microbiol. **59:**1213–1219.
22. **Lanka, S. T. J., M. Klein, U. Ramsperger, D. G. Müller, and R. Knippers.** 1993. Genome structure of a virus infecting the marine brown alga *Ectocarpus siliculosus*. Virology **193:**802–811.
23. **Mack, D. H., and J. J. Sninsky.** 1988. A sensitive method for the identification of uncharacterized viruses related to known virus groups: hepadnavirus model system. Proc. Natl. Acad. Sci. USA **85:**6977–6981.
24. **Mayer, J. A.** 1978. A lytic virus in *Micromonas pusilla*. Ph.D. thesis. University of British Columbia, Vancouver, Canada.
25. **Mayer, J. A., and F. J. R. Taylor.** 1979. A virus which lyses the marine nanoflagellate *Micromonas pusilla*. Nature (London) **281:**299–301.
26. **Meints, R. H., A. M. Schuster, and J. L. Van Etten.** 1985. *Chlorella* viruses. Plant Mol. Biol. Rep. **3:**180–187.
27. **Molina, F. I., L. M. Geletka, S. C. Jong, and Y. F. Zhang.** 1994. Use of a nested primer pair as control for PCR amplification of ribosomal DNA internal transcribed spacers in fungi. BioTechniques **16:**998–1001.
28. **Müller, D. G.** 1991. Marine virioplankton produced by infected *Ectocarpus siliculosus* (Phaeophyceae). Mar. Ecol. Prog. Ser. **76:**101–102.
29. **Müller, D. G., H. Kawai, B. Stache, and S. T. J. Lanka.** 1990. A virus infection in the marine brown alga *Ectocarpus siliculosus* (Phaeophyceae). Bot. Acta **103:**72–82.

30. **Müller, D. G., and B. Stache.** 1992. World-wide occurrence of virus infections in filamentous marine brown algae. Helgol. Meeresunters. **46:**1–8.
31. **Palenik, B.** 1994. Cyanobacterial community structure as seen from RNA polymerase gene sequence analysis. Appl. Environ. Microbiol. **60:**3212–3219.
32. **Palenik, B., and R. Haselkorn.** 1992. Multiple evolutionary origins of prochlorophytes, the chlorophyll b-containing prokaryotes. Nature (London) **355:**265–267.
33. **Paul, J. H., L. Cazares, and J. Thurmond.** 1990. Amplification of the *rbcL* gene from dissolved and particulate DNA from aquatic environments. Appl. Environ. Microbiol. **56:**1963–1966.
34. **Puig, M., J. Jofre, F. Lucena, A. Allard, G. Wadell, and R. Girones.** 1994. Detection of adenoviruses and enteroviruses in polluted waters by nested PCR amplification. Appl. Environ. Microbiol. **60:**2963–2970.
35. **Suttle, C. A.** 1994. The significance of viruses to mortality in aquatic microbial communities. Microb. Ecol. **28:**237–243.
36. **Suttle, C. A., and A. M. Chan.** Viruses infecting the marine prymnesiophyte *Chrysochromulina* spp.: isolation, preliminary characterization and natural abundance. Mar. Ecol. Prog. Ser., in press.
37. **Suttle, C. A., A. M. Chan, and M. T. Cottrell.** 1990. Infection of phytoplankton by viruses and reduction of primary productivity. Nature (London) **347:** 467–469.
38. **Suttle, C. A., A. M. Chan, and M. T. Cottrell.** 1991. Use of ultrafiltration to isolate viruses from seawater which are pathogens of marine phytoplankton. Appl. Environ. Microbiol. **57:**721–726.
39. **Tsai, Y.-L., M. D. Sobsey, L. S. Sangermano, and C. J. Palmer.** 1993. Simple method of concentrating enteroviruses and hepatitis A virus from sewage and ocean water for rapid detection by reverse transcriptase-polymerase chain reaction. Appl. Environ. Microbiol. **59:**3488–3491.
40. **Van Etten, J. L., D. E. Burbank, D. Kuczmarski, and R. H. Meints.** 1983. Virus infection of culturable *Chlorella*-like algae and development of a plaque assay. Science **219:**994–996.
41. **Van Etten, J. L., and S. A. Ghabrial.** 1991. Phycodnaviridae. Arch. Virol. **1991**(Suppl. 2)**:**137–139.
42. **Van Etten, J. L., L. C. Lane, and R. H. Meints.** 1991. Viruses and viruslike particles of eukaryotic algae. Microbiol. Rev. **55:**586–620.
43. **Wang, T. S.-F.** 1991. Eukaryotic DNA polymerases. Annu. Rev. Biochem. **60:**513–552.
44. **Waters, R. E., and A. T. Chan.** 1982. *Micromonas pusilla* virus: the virus growth cycle and associated physiological events within the host cells; host range mutation. J. Gen. Virol. **63:**199–206.
45. **Zehr, J. P., and L. A. McReynolds.** 1989. Use of degenerate oligonucleotides for amplification of the *nifH* gene from the marine cyanobacterium *Trichodesmium thiebautii*. Appl. Environ. Microbiol. **55:**2522–2526.
46. **Zhang, Y. P., D. E. Burbank, and J. L. Van Etten.** 1988. *Chlorella* viruses isolated in China. Appl. Environ. Microbiol. **54:**2170–2173.
47. **Zhang, Y. P., and C. A. Suttle.** 1994. Design and use of PCR primers for B-family DNA polymerase genes to detect and identify viruses and microbes, p. a–85. *In* ASLO & PSA 1994 Joint Meeting. American Society for Limnology and Oceanography, Lawrence, Kans.
48. **Zimmerman, K., K. Pischinger, and J. W. Mannhalter.** 1994. Nested primer PCR detection limits of HIV-1 in the background of increasing numbers of lysed cells. BioTechniques **17:**18–20.
49. **Zintz, C. B., and D. C. Beebe.** 1991. Rapid re-amplification of PCR products purified in low melting point agarose gels. BioTechniques **11:**158–162.